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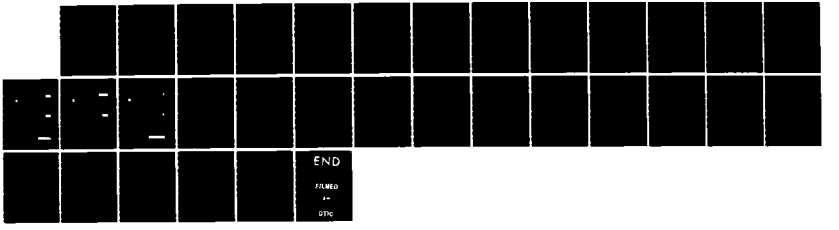

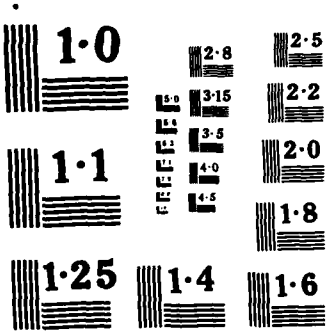

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ANIMAL MODELS FOR THE PREVENTION OF ACUTE AND CHRONIC GRAFT-VS-HOST DISEASE

Annual and Final Report

Michael T. Gallagher, Ph.D. and Lawrence D. Petz, M.D.

March 1984

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These studies considered the effect of monoclonal antibodies to several T-cell differentiation antigens on graft-versus-host disease in a murine model of bone marrow transplantation. The monoclonal antibodies were used for in vitro treatment of bone marrow and/or spleen cells prior to transplantation and were also used to treat established graft-versus-host disease by in vivo injections. The results increase our understanding of transplantation biology and can form the basis for clinical studies.

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SUMMARY

One of the purposes of the work was to develop a better understanding of the mechanism whereby pre-treatment of a mixture of bone marrow and spleen cells (which are used in a murine model of bone marrow transplantation) with rabbit antisera against murine lymph node tissue could effect not only acute graft-versus-host disease mortality but also chronic graft-versus-host disease mortality. The second major objective of the resesarch was to evaluate monoclonal antibodies to several T cell differentiation antigens both as agents for the in vitro pretreatment of bone marrow and spleen cell grafts to modify their graft-versus-host disease potential, and also as agents for the in vivo treatment of established graft-versus-host disease.

The methods used involved a well characterized murine model of bone marrow transplantation. In this model, the recipient mice are treated with lethal doses of total body irradiation. The injection of allogeneic bone marrow cells results in the development of chronic graft-versus-host disease in the recipient mice. In contrast, injection of mixtures of allogeneic bone marrow and spleen cells or spleen cells alone produces an immediate or acute form of graft-versus-host disease. In order to accomplish the above goals of the study, pretreatment of the allogeneic bone marrow and spleen cell grafts was carried out with either rabbit antisera against murine lymph node tissue or with a variety of monoclonal antibodies to T cell differentiation antigens. The effects of these in vitro treatments on acute and chronic graft-versus-host disease were monitored primarily by using lethal graft-versus-host disease as an endpoint.

The results indicated that pretreatment of allogeneic bone marrow cells with rabbit anti-mouse lymph node serum caused a marked reduction in chronic graft-versus-host disease mortality by day 60.

Studies with the monoclonal antibodies were initiated by first determining their cytotoxicity in vitro, as measured by a trypan blue exclusion test. The cytotoxicity titers were in excess of 1,000 to 3,000. However, when the antibodies were used in these dilutions to treat mixtures of murine bone marrow and spleen cells, there was no signficant inhibition of graft-versus-host disease in the recipient animals. At dilutions of 1:300 however, anti-THY 1.2, anti-LYT 1.2 and anti-LYT 2.2 did show consistent inhibition of acute graft-versus-host disease. However, anti-THY 1.1 was ineffective in this regard.

Tests with monoclonal antibodies of the lymphocytes of allogeneically reconstituted mice indicated no marked differences in T cell populations between mice constituted with bone marrow that was not treated in vitro compared with allogeneically reconstituted mice where the bone marrow was treated in vitro

with rabbit anti-lymph node serum. This result argues for the complete elimination of lymphoid elements as a mechanism whereby in vitro treatment with rabbit anti-lymph node serum of allogeneic bone marrow can reduce the incidence of chronic graft-versus-host disease mortality.

Studies of the effect of a single in vivo treatment with monoclonal antibody four days post-transplant on mortality from established acute graft-versus-disease indicated that treatment with anti-LYT 1.2 was effective in reducing mortality from 100% to 50% at 21 days and with a prolongation of mean survival time from 10 days to 19.6 days. However, similar experiments performed with anti-LYT 2.2 and anti-THY 1.2 revealed no significant difference in mortality or in mean survival time of the recipient mice. Multiple treatments with monoclonal antibodies against T-cell differentiation antigens on days 1, 3, and 7 post-transplant had no effect on mortality from established acute or chronic graft-versus-disease.

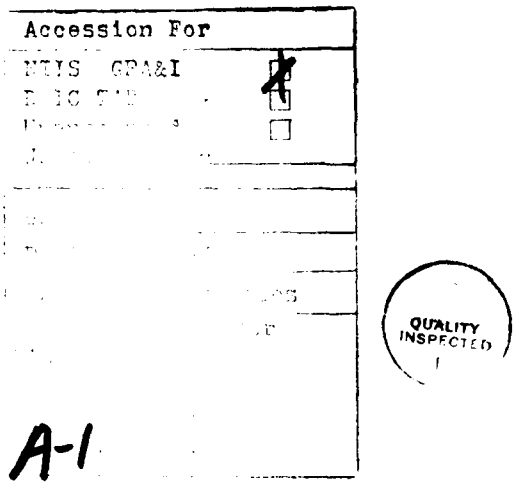
The conclusions reached from these studies are that complete elimination of lymphoid elements (both mature and immature), rather than selective removal is the mechanism whereby rabbit anti-mouse lymph node serum has its effect on chronic graft-versus-host disease mortality in our murine bone marrow transplantation model.

Further, in vitro treatment of bone marrow cells or bone marrow and spleen cells with low concentrations of monoclonal antibodies had no signficant effect on mortality or mean survival time. However, pre-treatment of bone marrow or bone marrow and spleen cells with a higher concentration of certain monoclonal antibodies to T-cell differentiation antigens did prolong survival and decrease mortality from graft-versus-host disease. Treating established graft-host-disease with in vivo injections of monoclonal antibodies to T-cell differentiation antigens had little effect on established acute or chronic graft-versus-host disease, except in one experiment using anti-LYT 1.2.

These findings increase our understanding of transplantation biology and can form the basis for clinical studies which would have as their aim the decrease of graft-versus-host disease by in vitro depletion of T-cells or subsets of T-cells in the bone marrow of the donor prior to transplantation.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No.(NIH) 78-23, Revised 1978).



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We have made substantial progress towards realizing the objectives of our contract entitled "Animal Models for the Prevention of Acute and Chronic Graft-Versus-Host Disease". The objectives of our research were two-fold. First, we designed experiments which would allow us to better understand the mechanism(s) whereby a single in vitro pretreatment of a bone marrow and spleen cell graft with rabbit antisera made against murine lymph node tissue could effect not only acute graft-versus-host disease (GVHD) mortality but also late developing chronic GVHD mortality. The understanding of this phenomenon could lead to better methods of prophylaxis and treatment of chronic GVHD in the clinic.

Our second major objective was to evaluate monoclonal antibodies to several T-cell differentiation antigens as potential agents for the in vitro pretreatment of murine bone marrow and spleen cell grafts to modify their GVHD potential.

Both objectives have the same overall goal of increasing the number of patients who could benefit from bone marrow transplantation by elimination or modifying GVHD which is the major obstacle to a more widespread use of allogeneic bone marrow transplantation today. Militarily this would make bone marrow transplantation a more viable therapy in the event of nuclear accidents or limited nuclear war.

I. A Study of the Incidence of Chronic GVHD Mortality in Mice Receiving Mixtures of Bone Marrow and Spleen or Bone Marrow Cells Alone Pretreated with Rabbit Antimouse Antisera

These experiments were designed to evaluate the effect of a single pretreatment with rabbit antimouse lymph node sera (RAMLNS) on the chronic GVHD potential of murine bone marrow grafts. We have previously shown that numerous rabbit antimouse antisera are capable of reducing the acute GVHD potential of mixtures of murine bone marrow and spleen cells. In these experiments, we also reported that mice receiving spleen and bone marrow cells that had been pretreated with rabbit antisera to mouse lymph node cells (RAMLNS and RAMPLNS) showed a greatly reduced incidence of chronic GVHD mortality (Table I). How a single in vitro pretreatment could effect the incidence of chronic GVHD mortality was a puzzle. Chronic GVHD in the murine model is thought to be the result of recognition of host antigens by immune competent cells derived from stem cells in the initial inoculum. How could a single pretreatment effect cells not present at the time of the treatment? Our initial observations were made in mice receiving pretreated mixtures of bone marrow and spleen cells. If the effect of the RAMLNS was due to complete elimination of lymphoid elements (both mature and immature), one could expect chronic GVHD mortality to be the same in mice receiving treated mixtures of bone marrow and spleen cells or treated bone marrow cells alone. However, if alteration of mature T-cell subpopulations in the graft was involved (i.e., killing of helper T-cells while sparing suppressor T-cells), one might expect the antibodies to be effective on mixtures of bone marrow and spleen but not on bone marrow cells alone. These results would be expected because bone marrow and spleen cell mixtures contain large numbers of T-cells (thus, various subpopulations), whereas bone marrow cells alone have almost no mature T-cells.

TABLE I

THE EFFECT OF RABBIT ANTIMOUSE LYMPH NODE SERA ON THE ACUTE AND CHRONIC GVHD POTENTIAL OF MURINE SPLEEN AND BONE MARROW CELLS AT BAYLOR COLLEGE OF MEDICINE

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 14 Day (Acute GVHD)	% Mortality 90 Day (Chronic GVHD)
$(C57XA)_{f1}$	18	A BM & SPLEEN	100%	--
$(C57XA)_{f1}$	18	A BM & SPLEEN R_x RAMMLNS	0%	5%
$(C57XA)_{f1}$	18	A BM & SPLEEN R_x RAMPLNS	0%	0%

Initially, we tested the RAMLNS (RAMMLNS and RAMPLNS) for effectiveness in an acute GVHD model. THe results of these experiments are shown in Table II. This table presents the combined results of four separate experiments. Twenty-nine lethally irradiated mice receiving no graft died within 14 days of aplasia. This irradiation control group served to verify that the mice were indeed lethally irradiated. In the cell control group, again, 29 of 29 $(C57XA)_{f1}$ hybrid mice which were lethally irradiated and injected with $5X10^7$ untreated A strain bone marrow and spleen cells died by day 14. This group died of acute GVHD not of aplasia as did the irradiation controls. The third group died of acute GVHD not of aplasia as did the irradiation controls. The third group received mixtures of A strain bone marrow and spleen cells that had been pretreated in vitro with RAMPLNS. In this group, acute GVHD mortality was reduced from 100% to 50%. Finally, group 4 received mixtures of bone marrow and spleen cells pretreated with RAMMLNS. In this group, the acute GVHD mortality was only 20%. The experiments demonstrated that the RAMLNS were still highly effective in abrogation of acute GVHD mortality. The differences in the percent acute GVHD mortality seen in the treated groups in the present experiments and our previously published results (Table I) were not unexpected since the previously reported data were obtained in a barrier sustained specific pathogen free (SPF) mouse colony at Baylor College of Medicine, whereas the data obtained at the City of Hope National Medical Center used commercially obtained SPF mice housed in a clean conventional mouse colony. It is well known that GVHD mortality is complicated by many factors and that mortality is usually higher in conventional colonies.

Having established the basic model and the effectiveness of the RAMLNS in our laboratory, we then proceeded to test the hypothesis outlined above. THe results of several experiments measuring the effects of a single in vitro pretreatment with RAMLNS on the chronic GVHD mortality produced by bone marrow cells alone is presented in Table III. Thirty-six mice receiving lethal irradiation and 10^7 untreated A strain bone marrow cells showed 70% chronic GVHD mortality by day 60. Nineteen $(C57XA)_{f1}$ hybrids receiving lethal irradiation and 10^7 A strain bone marrow cells pretreated in vitro with RAMLNS showed only 16% chronic GVHD mortality in the same time period. In short, a single in vitro pretreatment of bone marrow cells with RAMLNS resulted in a greatly reduced chronic GVHD mortality. This result supports the hypothesis that complete elimination of lymphoid elements (both mature and immature), not selective removal or concentration of a particular mature T-cell subpopulation is the mechanism whereby RAMLNS has it effect of chronic GVHD mortality.

II. The Use of Monoclonal Anti-T-Cell Antibodies to Characterize the T-Cell Populations of Mice Receiving Pretreated Murine Cell Grafts

In addition to the experiments reported above, we have also used monoclonal antibodies against various T-cell antigens (THY 1.2, LYT 1.2, LYT 2.2) to characterize the T-cell populations in lymphoid organs of $(C57XA)_{f1}$ hybrid mice receiving A strain bone marrow cells either untreated or pretreated with RAMLNS in vitro. Syngeneically reconstituted mice were used as controls in these experiments. Again, these experiments are directed towards a better understanding of the mechanism(s) by which a single in vitro pretreatment with RAMLNS can effect chronic GVHD mortality. If alteration of T-cell balance is involved (i.e., increased suppressor-decreased helper), one might expect differences in T-cell subpopulations in mice

TABLE II

THE EFFECT OF RABBIT ANTIMOUSE LYMPH NODE SERA ON THE ACUTE GVHD POTENTIAL OF MURINE SPLEEN AND BONE MARROW CELLS AT CITY OF HOPE NATIONAL MEDICAL CENTER

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 14 Day (Acute GVHD)
$(C57XA)_{f1}$	29	None	100%
$(C57XA)_{f1}$	29	A BM & SPLEEN	100%
$(C57XA)_{f1}$	24	A BM & SPLEEN R_x RAMPLNS	50%
$(C57XA)_{f1}$	24	A BM & SPLEEN R_x RAMMLNS	20%

TABLE III

THE EFFECT OF A SINGLE IN VITRO PRETREATMENT WITH RABBIT ANTIMOUSE LYMPH NODE SERA ON THE CHRONIC GVHD POTENTIAL OF MURINE BONE MARROW CELLS

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 60 Day (Chronic GVHD)
$(C57XA)_{f1}$	36	A BM	70%
$(C57XA)_{f1}$	19	A BM R_x RAMLNS	16%

receiving treated or untreated cells. However, if complete removal of lymphoid elements is the mechanism whereby a single treatment is effective, differences in T-cell subpopulations might not be observed. The results of experiments measuring T-cell subpopulations in normal A strain mice, autologously repopulated A strain mice, $(C57XA)_{f1}$ hybrids repopulated with untreated A strain cells and $(C57XA)_{f1}$ hybrids repopulated with RAMLNS treated A strain cells are presented in Figures 1, 2, and 3. These figures show data from approximately 90 days post-transplant. The data presented here is given in terms of specific cytotoxicity (i.e., the cytotoxicity seen when normal sera and complement are used is subtracted from the cytotoxicity seen when the antibody and complement is used). Figure 1 shows the cytotoxicity of anti-THY 1.2 monoclonal antibody against thymus, spleen, and lymph node cells of the 4 groups of mice under study. All irradiation chimeras have more cells in the thymus which can be killed by anti-THY 1.2 and complement than do the normal controls. This may reflect the increased lymphocyte production needed to repopulate the tissues of lethally irradiated mice. However, there was no significant differences seen in the number of THY 1.2 bearing cells in the allogeneically reconstituted mice receiving untreated marrow (ALLOBM) and the allogeneically reconstituted mice receiving bone marrow pretreated with RAMLNS in vitro ($ALLOBMR_X$).

In the spleen there were no differences seen in the THY 1.2 population in any of the four groups of mice studied Finally, in the lymph node, all irradiation chimeras showed lower numbers of THY 1.2 sensitive cells possibly reflecting incomplete reconstitution of the peripheral lymphoid system. Again, there were no significant differences seen between the ALLOBM mice and the $ALLOBMR_X$ mice. Figure 2 shows the cytotoxicity of anti-LYT 1.2 monoclonal antibody against thymus, spleen and lymph node cells of the four groups of mice. In the thymus, normal and autologously reconstituted mice had undetectable levels of LYT 1.2 bearing cells, while both allogeneically reconstituted groups showed low levels of LYT 1.2 bearing cells (4% and 6.3%). This again did not represent a significant difference in ALLOBM and $ALLOBMR_X$ mice. In the spleen, a small but insignificant difference was observed in that ALLOBM mice had no detectable LYT 1.2 bearing cells whereas $ALLOBMR_X$ mice had 2.5% LYT 1.2 bearing cells. In the lymph nodes, ALLOBM and $ALLOBMR_X$ mice had 6% and 8.8% LYT 1.2 positive cells whereas normal or autologously reconstituted mice had 3.5% and 4%.

Figure 3 shows the cytotoxicity of LYT 2.2 monoclonal antibodies against thymus, spleen and lymph node cells of normal, autologously reconstituted ALLOBM and $ALLOBMR_X$ mice. All irradiation chimeras showed higher than normal levels of LYT 2.2 bearing cells in the thymus, however, there were no significant differences between the ALLOBM and $ALLOBMR_X$ groups. In the spleen, all mice had low levels of detectable LYT 2.2 bearing cells. Finally, in the lymph nodes all mice showed low levels of LYT 2.2 positive cells with the $ALLOBMR_X$ group having the highest value at approximately 5%. THY 1 is a pan T-cell antigen of mice that is present in high concentration on mouse thymocytes and in lower concentration on peripheral T-cells. Most mice have the THY 1.2 allele as do the A strain mice used in our study. The LYT 1 antigen is used to mark the helper T-cells in our study. We use the LYT 2 antigen to mark the suppressor/cytotoxic T-cell compartment. The helper T-cell population in mice is actually marked with both LYT 1 and LYT 2 antigens. The cytotoxic/suppressor T-cell population is marked with LYT 2 and LYT 3 antigens. Thus, LYT 2 antisera should kill helper and cytotoxic/suppressor T-cells. On the other hand, LYT-1 antisera should kill only

FIGURE 1. % THY 1.2 BEARING CELLS IN:

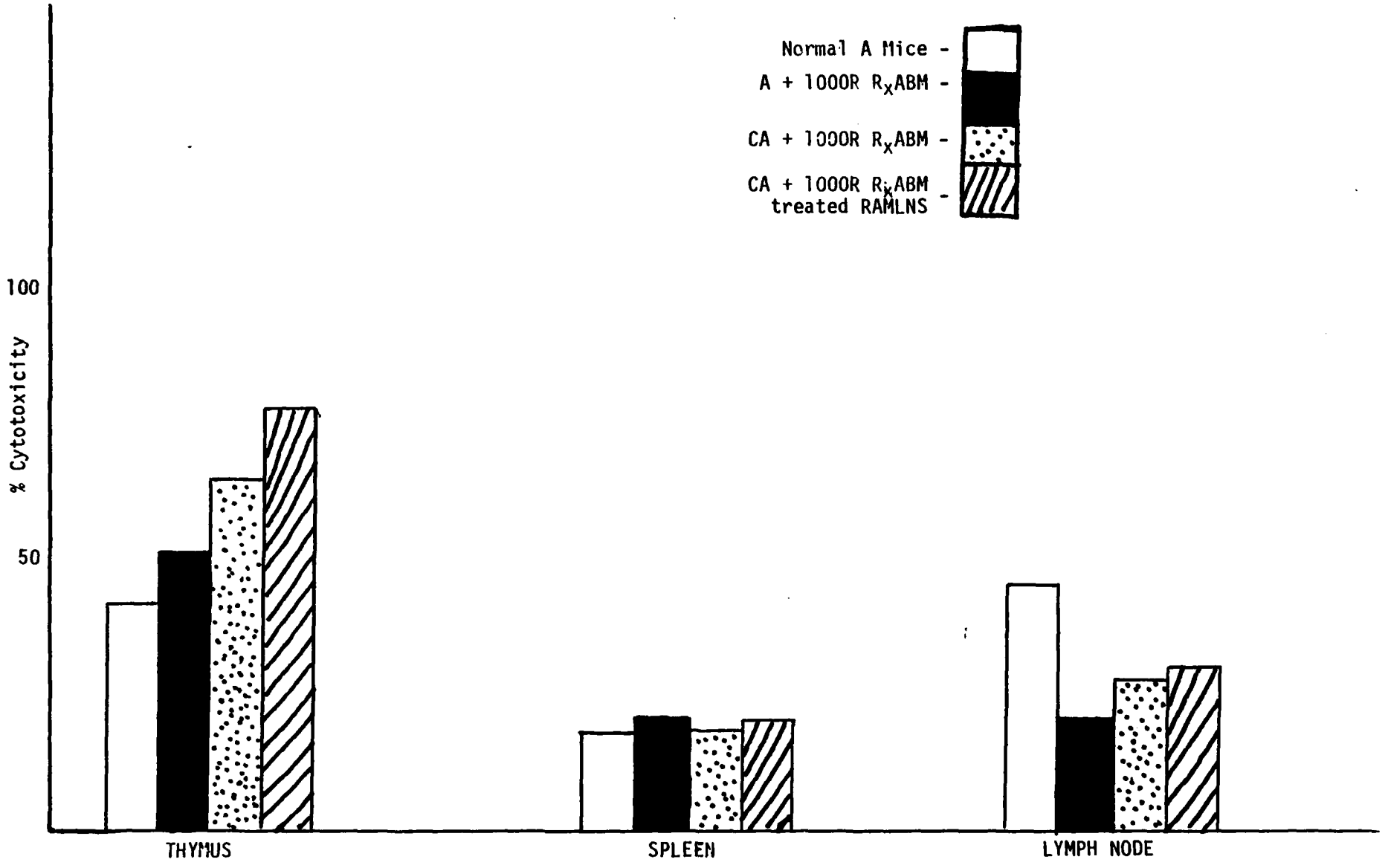


FIGURE 2. % LYT 1.2 BEARING CELLS IN:

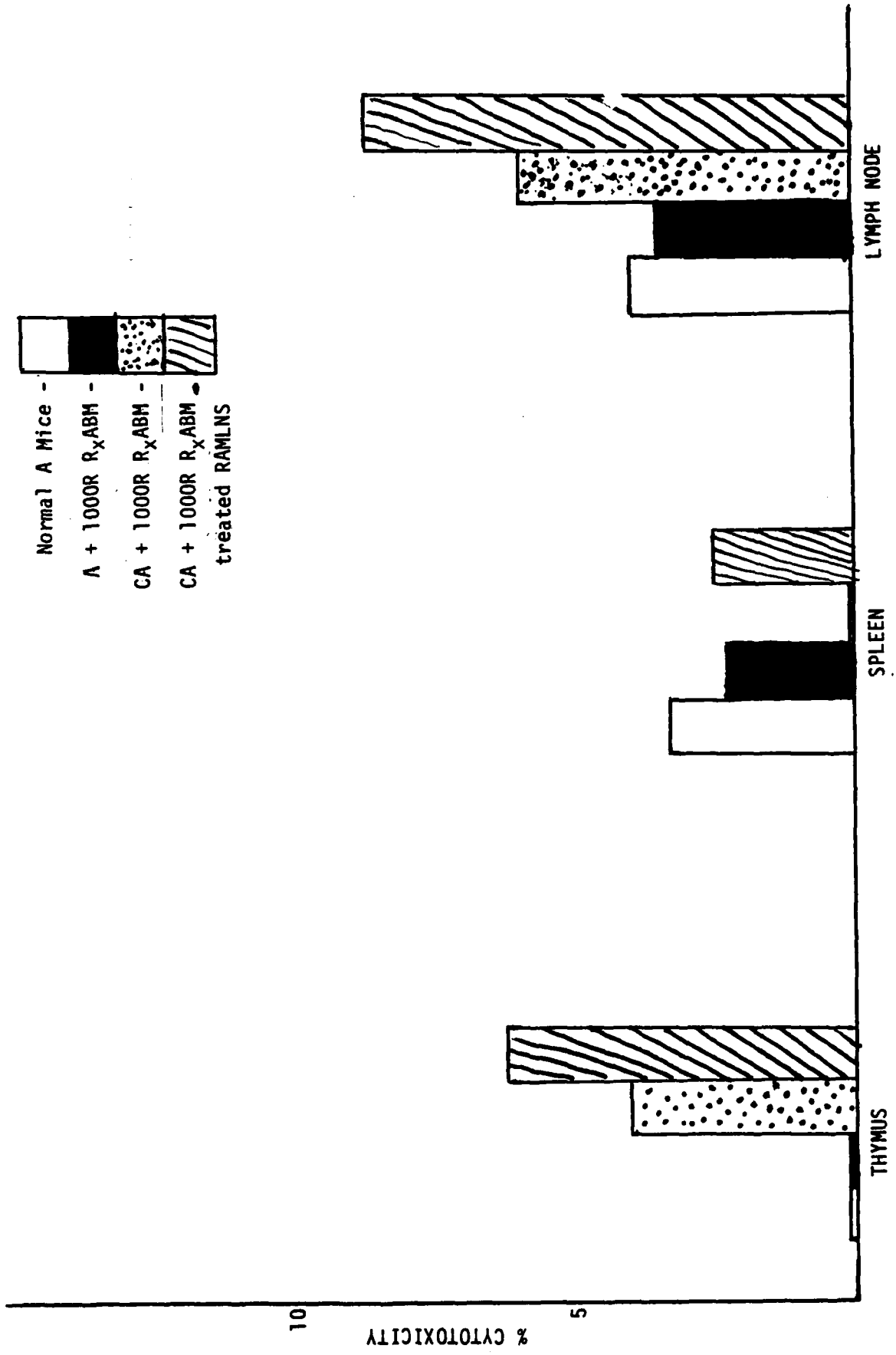
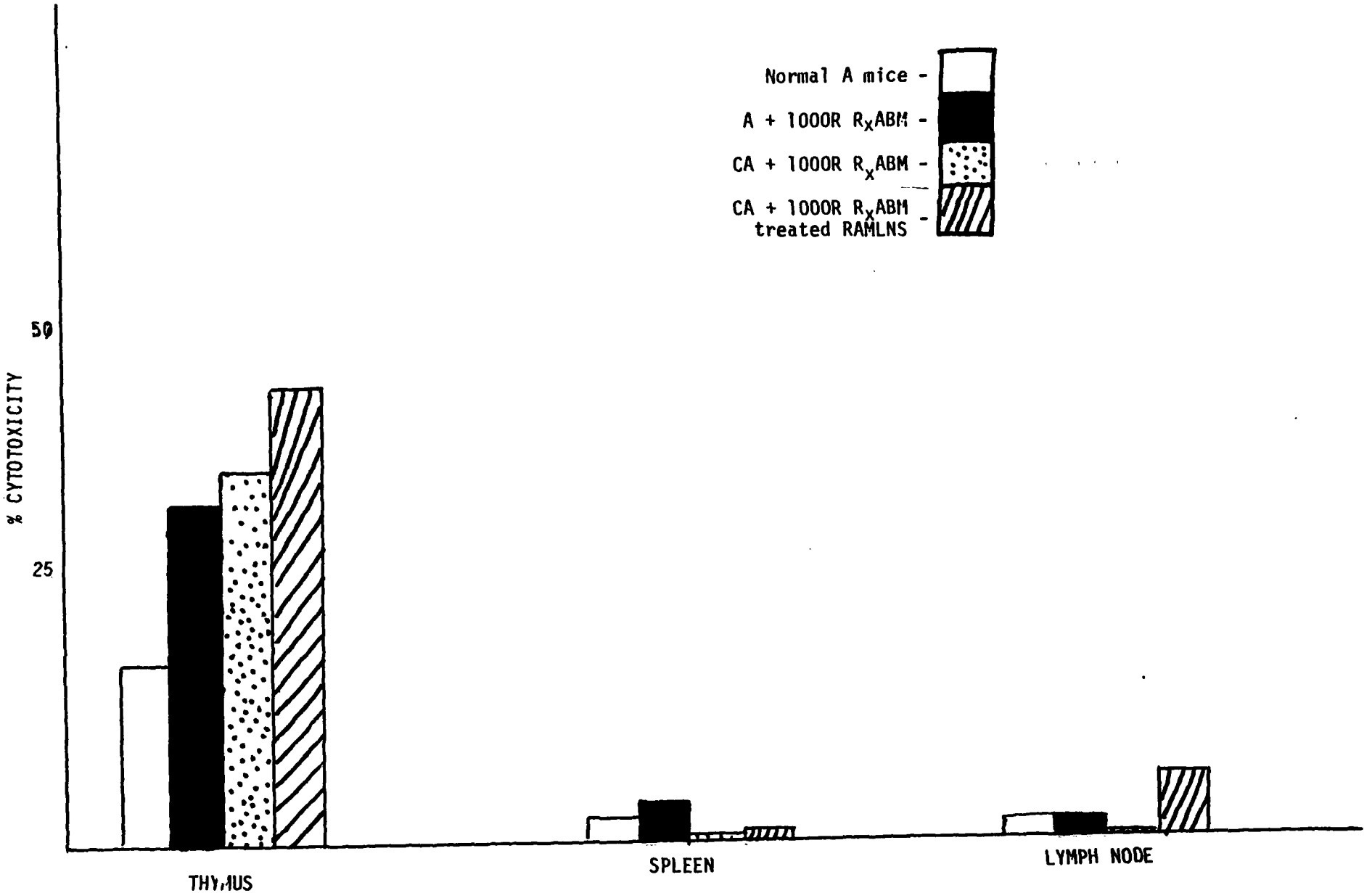


FIGURE 3. LYT 2.2 BEARING CELLS IN:



helper T-cells. This allows for calculation of cytotoxic/suppressors by subtracting the LYT 1 positive cells from the LYT 2 positive cells. When these calculations are made, again no differences are seen in the ALLOBM and the ALLOBMR$_x$ mice. The results presented here represent a minimum of four mice per point. Similar studies at times after 90 days also reveal no differences in the T-cell subpopulations of ALLOBM and ALLOBMR$_x$ mice.

The overall result again argues for the complete elimination of lymphoid elements as the mechanism whereby RAMLNS can, with one initial pretreatment effect the incidence of chronic GVHD mortality. Thus, it appears that if all lympoid elements, both mature and immature can be removed from a graft not only will acute GVHD be modified but also substantial effects on chronic GVHD can be realized. Thus, the search for agents which can eliminate lymphoid elements from grafts must be continued.

III. The Effect of In Vitro Pretreatment With Monoclonal Antibody Directed Against Various T-Cell Antigens on the Acute Graft-Versus-Host Disease Potential of Mixtures of Murine Spleen and Bone Marrow Cells

As we and others have shown, conventional whole antisera can be very effective agents for the modifications of the GVHD potential of a graft. However, there are problems with the application of conventional whole antisera techniques to clinical bone marrow transplantation. First, every batch of conventional whole antisera will have different properties. Second, the absorption procedures necessary are complex and time consuming. Finally, there is no assay available for directly measuring the effect of an antisera on human pluripotent stem cells. Again, what is needed for in vitro elimination of GVHD potential is an agent which is highly specific for T-cells with no cross-reactivity for stem cells. We feel that monoclonal antibodies directed against T-cell antigens are good candidates for future clinical use in these systems. Monoclonal antibodies would eliminate the problems of non-uniform sera. In addition, absorption would be unnecessary. Finally, monoclonal antibodies are exquisitely specific and therefore should offer no problems of cross-reactivity with stem cells. For these reasons, we evaluated monoclonal antibodies to several T-cell antigens as potential agents for the in vitro pretreatment of murine bone marrow and spleen cell grafts to modify their GVHD potential.

The first step in these studies was to evaluate the in vitro cytotoxicity of the monoclonal antibodies to be studied with our complement source. This was done using a trypan blue exclusion cytotoxicity test. The monoclonal antibodies employed in this study were obtained from New England Nuclear, or Becton Dickinson. Table IV shows the in vitro cytotoxicity of anti-THY 1.1 and THY 1.2 monoclonal antibodies on lymphoid cells from A and (C57XA)$_{f1}$ hybrid mice. Both the A strain and the (C57XA)$_{f1}$ hybrid mice are THY 1.2 positive, therefore the anti-THY 1.1 monoclonal antibody serves as a negative control. The anti-THY 1.1 antibody plus complement (C) produced low levels of lysis in all cells tested (8-14%). On the other hand, anti-THY 1.2 plus complement killed 86% of A thymocytes, 82% of (C57XA)$_{f1}$ thymocytes and 41% of (C57XA)$_{f1}$ splenocytes at a 1/3000 dilution. The results showed that the anti-THY 1.2 monoclonal was very effective with our source of complement. The other monoclonal antibodies to be used in our experiments were also tested in this manner and shown to be effective with our complement source all showing in vitro cytotoxicity titres of >1:3000.

TABLE IV

THE IN VITRO CYTOTOXICITY OF MONOCLONAL ANTI THY 1.1 AND THY 1.2 ON LYMPHOID CELLS FROM A AND $(C57XA)_{f1}$ MICE (1/3500 Dilution)

Monoclonal Antibody	% Cytotoxicity To: A Thymus	$(C57XA)_{f1}$ Thymus	$(C57XA)_{f1}$ Spleen
THY 1.1 + C	14%	8%	10%
THY 1.2 + C	86%	82%	41%

Table V shows a summary of several experiments measuring the effects of in vitro pretreatment with monoclonal antibodies against T-cell antigens on the acute GVHD potential of murine spleen and bone marrow cells. In these initial experiments, monoclonal antibody to T-cell antigens was added to mixtures of bone marrow and spleen cells in vitro. The mixtures were incubated for 30 minutes at 37°C, then injected into lethally irradiated recipients. In these experiments, no complement was added in vitro since when using whole antisera in this type of experiment, the complement present in the irradiated recipient is sufficient to kill the sensitized cells of the graft. However, as can be seen, the monoclonal antibodies used (THY 1.2, LYT 1.2, and LYT 2.2) were completely ineffective without complement in vitro. All mice receiving bone marrow and spleen cells pretreated with monoclonal antibody in vitro died within 14 days of acute GVHD as did the untreated cell controls. After these initial experiments, all treatments included the addition of complement in vitro. Table VI shows the combined results of several experiments in which a single large inoculum (enough cells for six transplants) was treated in vitro with monoclonal antibody and complement. (The final dilution of the monoclonal antibodies used in these experiments was 1:3000. This treatment technique was employed in all of our previous work with whole antisera.) Again, all mice receiving lethal irradiation and untreated cells were dead of acute GVHD by day 14. The mice receiving cells pretreated with THY 1.2 plus complement showed a slight reduction in mortality with 4 of 18 mice surviving more than 14 days. However, the groups receiving mixtures of bone marrow and spleen cells pretreated with LYT 1.2 or LYT 2.2 and complement showed no prolonged survival. These results are somewhat paradoxical with monoclonal antibody plus complement killing T-cells in vitro (Table IV), but with only minimal modification of acute GVHD mortality seen (Table VI). Some possible problems with the use of monoclonal antibody in these experiments may relate to unusually low avidity for the antigen. This can usually be minimized by treatment of the cells with antibody at 4°C, centrifuging and resuspending in media containing complement. This technique was employed in our experiments to no avail. The final dilution of monoclonal antibody used in our initial experiments was 1/3000. The cytotoxic titer of the antibodies used in these experiments was approximately 1/5000; therefore, we were well within the dilution limits of our antibodies. Finally, clumping of lymphoid cells during the treatment period could result in protection of lymphoid cells. Thinking that this phenomenon could result in making our pretreatments with monoclonal antibody less effective, we designed experiments in which smaller aliquots of bone marrow and spleen cell mixtures (enough cells for one transplant) were treated. In these experiments, the cell concentration was lower and the chances of clumping therefore, decreased. The final dilution of monoclonal antibody was 1:1000. The combined results of several experiments done in this manner are presented in Table VII. Forty mice receiving untreated cells (cell controls) all died by day 14 of acute GVHD. In 24 mice receiving cell mixtures pretreated with monoclonal anti-THY 1.2 and complement acute GVHD mortality was reduced to 75%. In six mice receiving cell mixtures pretreated with monoclonal ant-LYT 1.2 plus complement acute GVHD mortality was reduced to 33%. Finally, in 13 mice receiving cell mixtures pretreated with monoclonal anti-LYT 2.2 plus complement acute GVHD mortality was reduced to 47%.

In a final series of experiments small aliquots of bone marrow and spleen cells were treated with various monoclonal antibodies at a final

TABLE V

THE EFFECT OF IN VITRO PRETREATMENT WITH MONOCLONAL ANTIBODIES ALONE ON THE ACUTE GVHD POTENTIAL OF MURINE BONE MARROW AND SPLEEN CELLS

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 14 Day (Acute GVHD)
$(C57XA)_{f1}$	11	A BM & SPLEEN	100%
$(C57XA)_{f1}$	12	A BM & SPLEEN R_x THY 1.2	100%
$(C57XA)_{f1}$	12	A BM & SPLEEN R_x LYT 1.2	100%
$(C57XA)_{f1}$	12	A BM & SPLEEN R_x LYT 2.2	100%

TABLE VI

THE EFFECT OF IN VITRO PRETREATMENT WITH MONOCLONAL ANTIBODY PLUS COMPLEMENT ON THE ACUTE GVHD POTENTIAL OF MURINE BONE MARROW AND SPLEEN CELLS (1:3000 Dilution)

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 21 Day (Acute GVHD)
$(C57XA)_{f1}$	16	A BM & SPLEEN	100%
$(C57XA)_{f1}$	18	A BM & SPLEEN R_x THY 1.2 + C'*	88%
$(C57XA)_{f1}$	12	A BM & SPLEEN R_x LYT 1.2 + C'	100%
$(C57XA)_{f1}$	12	A BM & SPLEEN R_x LYT 2.2 + C'	100%

* Complement

TABLE VII

THE EFFECT OF IN VITRO PRETREATMENT WITH MONOCLONAL ANTIBODY PLUS COMPLEMENT ON THE ACUTE GVHD POTENTIAL OF MURINE BONE MARROW AND SPLEEN CELLS (1:1000 Dilution)

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 21 Day (Acute GVHD)
$(C57XA)_{f1}$	40	A BM & SPLEEN	100%
$(C57XA)_{f1}$	24	A BM & SPLEEN R_x THY 1.2 + C'*	75%
$(C57XA)_{f1}$	6	A BM & SPLEEN R_x LYT 1.2 + C'	33%
$(C57XA)_{f1}$	13	A BM & SPLEEN R_x LYT 2.2 + C'	47%

* Complement

dilution of 1:300. The results are presented in Table VIII. At a final dilution of 1:300 monoclonal antibody to THY 1.2 reduces acute GVHD mortality from control levels of 100% to 17% while monoclonal antibody to LY 1.2 and LY 2.2 reduces acute GVHD mortality to 0 and 16% respectively. Table VIII also shows the result of treatment of mixtures of AKR bone marrow and spleen cells with monoclonal antibody directed against the THY 1.1 antigen. This antibody has an in vitro cytotoxicity titer of >1:5000, however even at a low dilution of 1:300 it is essentially ineffective in our model reducing the mortality at 21 days post-transplant to only 92%. Finally, Table VIII shows additional experiments using RAMLNS as an agent for the pretreatment of bone marrow and spleen cell grafts to reduce their acute GVHD potential. Over 90% of the lethally irradiated $(C57XA)_{f1}$ mice receiving either A or AKR cells pretreated with RAMLNS survived acute GHVD.

Other groups have reported the successful use of monoclonal anti-THY 1.2 pretreatment to eliminate or reduce the acute GVHD potential of mixtures of murine bone marow and spleen cells. In one system this antibody was effective in reducing GVHD mortality in C57 mice lethally irradiated and injected with mixtures of BALB/c bone marrow and spleen cells. The GVHD model employed in these studies appeared to be weak since only 30% of the mice receiving untreated cells died by day 14. The 60 day mortality in their control group was 90%. Nevertheless, they showed reduction of 60 day mortality from the control level of 90% to 20% in mice receiving the cell mixtures which were pretreated with monoclonal anti-THY 1.2 and complement. Thierfelder et al have recently reported that monoclonal antibodies directed against the THY 1.2 and THY 1.1 antigen were effective in vitro pretreatment agents in their murine model. Again the GVHD model used was somewhat weak since no mice receiving untreated spleen and bone marrow cells died by day 30. The authors reported a reduction in 50 day mortality from 100% (10/10) to 0% (0/10) in $(C57BL \times CBA)_{F1}$ hybrid mice receiving untreated or anti-THY 1.2 treated bone marrow and spleen cells respectively. In another model (C57BL into CBA) these authors report a reduction in 50 day mortality from 100% (12/12) to 44% (4/9) by pretreatment of the C57BL bone marrow and spleen cell grafts with anti THY 1.2.

In our strong acute GVHD model where all mice receiving untreated spleen and bone marrow cells die by day 21 monoclonal antibodies directed against THY 1.2, LYT 1.2 and LYT 2.2 have been shown to be efective pretreatment agents. However, for maximum effectiveness high concentrations of the monoclonal antibodies in pretreatment mixture are required. The 4 monoclonal antibodies tested here were anti-THY 1.2, -LYT 1.2, -LYT 2.2 and -THY 1.1. All of these monoclonal antibodies when combined with complement have high cytotoxicity titers for appropriate murine T-cells as measured by dye exclusion. The cytotoxicity titers for these antibodies are all greater than 1:5000. However, when the antibodies are used at final dilutions of 1:3000 or 1:1000 to treat mixtures of murine bone marrow and spleen cells consistent inhibition of acute GVHD potential is not seen. When monoclonal antibodies to the T-cell antigens THY 1.2, LYT 1.2 and LYT 2.2 are used at final dilutions of 1:300 consistent inhibition of acute GVHD potential is seen. Another monoclonal antibody directed against the THY 1 antigen THY 1.1 is ineffective in inhibiting the acute GVHD potential of mixtures of AKR bone marrow and spleen cells at a final dilution of 1:300 even though it has essentially the same in vitro cytotoxocity pattern as monoclonal anti-THY 1.2 (both titers >1:5000). In both the A into $(C57 \times A)_{F1}$ and AKR into $(C57 \times A)_{F1}$ acute GVHD models conventional antisera made against mouse

TABLE VIII

THE EFFECT OF IN VITRO PRETREATMENT WITH MONOCLONAL ANTIBODY PLUS COMPLEMENT ON THE ACUTE GVHD POTENTIAL OF MURINE BONE MARROW AND SPLEEN CELLS (1:300 Dilution) COMPARED WITH THE EFFECTIVENESS OF WHOLE RABBIT ANTI-MOUSE LYMPH NODE SERUM (RAMLNS)

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 21 Day (Acute GVHD)
$(C57XA)_{f1}$	48	A or AKR BM & SPLEEN	100%
$(C57XA)_{f1}$	12	A or AKR BM & SPLEEN R_x Complement Alone	100%
$(C57XA)_{f1}$	24	A or AKR BM & SPLEEN R_x RAMLNS + C'*	8%
$(C57XA)_{f1}$	18	A BM & SPLEEN R_x THY 1.2 + C'	17%
$(C57XA)_{f1}$	6	A BM & SPLEEN R_x LYT 1.2 + C	0%
$(C57XA)_{f1}$	6	A BM & SPLEEN R_x LYT 2.2 + C	16%
$(C57XA)_{f1}$	24	AKR BM & SPLEEN R_x THY 1.1 + C	92%

* Complement

lymph node cells in rabbits (RAMLNS) were very effective in reducing the acute GVHD potential of murine lymphoid cells by in vitro pretreatment. These experiments point out two important factors. First, a high in vitro cytotoxicity titer does not always correlate with effectiveness of the agent for pretreatment of cell mixtures for the modification of acute GVHD potential. Second, monoclonal antibodies are not always superior to conventional antisera in reducing acute GVHD after a single in vitro pretreatment.

The decreased effectiveness of monoclonal antibodies in modifying the GVHD potential of murine lymphoid cells compared to their in vitro cytotoxocity for these cells as measured by dye exclusion may relate to the avidity of complement fixing ability of a given monoclonal antibody, or it may involve interference with the monoclonal antibody's activity by non T-cell present in the graft. When performing in vitro cytotoxocity tests pure populations of target cells are usually employed. However, when pretreating cell mixtures to modify their acute GVHD potential the target cells (T-cells) represent only a small minority of the cells present. Interference could be caused by non-specific absorption of antibody onto non T-cells of the graft or by specific removal of monoclonal antibody by cells possessing Fc receptors. Whatever the mechanism, a more complete understanding of what happens when mixtures of cells are treated with monoclonal antibody may help us to optimize the effetiveness of these agents in preventing GVHD. One way to increase the effectiveness of monoclonal antibodies as agents for the in vitro pretreatment of bone marrow grafts may be to utilize the specificity of the monoclonal antibody without relying on its cytotoxic potential.

We have also investigated the effectiveness of monoclonal antibodies to T-cell antigens as agents to treat established acute GVHD.

The effect of a single treatment with monoclonal antibody 4 days post-transplant on acute GVHD mortality

Table IX shows the results of three separate experiments using monoclonal antibodies to treat already established acute GVHD. In experiment 1 mice treated with monoclonal antibody directed against the T-cell antigen LYT 1.2 showed 50% mortality at 21 days and a mean survival time of 19.6 ± 8.9 days while mice receiving no treatment post-transplant showed 100% mortality and a mean survival time of 10.0 days. In experiment 2 monoclonal antibody against THY 1.2 and LYT 2.2 were used for post-transplantation treatment. Mortality was 100% in both cases with mean survival time of 10.7 ± 3.5 and 9.5 ± 1.5 respectively compared to a mean survival time of 11.3 ± 4.5 days for the untreated controls. In experiment 3 monoclonal antibody against THY 1.2 was again employed. Again no reduction in mortality was seen and the mean survival times between control and treatment groups were not significantly different. Thus, it appears that a single treatment with monoclonal antibody to LYT 1.2 can have an effect on mortality from already established acute GVHD. Treatment with monoclonal anti THY 1.2 or LYT 2.2 seems to be ineffective in our model.

The effect of multiple treatments with monoclonal antibody directed against T-cell antigens on mortality from already established acute GVHD

Table X again shows the results of 3 experiments. In experiment 1 nine mice receiving bone marrow and spleen cells and no treatment post-

TABLE IX

THE EFFECT OF A SINGLE IN VIVO TREATMENT WITH MONOCLONAL ANTIBODY FOUR DAYS POST-TRANSPLANT ON MORTALITY FROM ESTABLISHED ACUTE GVHD

Experiment	Group*	21 Day Mortality (Acute GVHD)	Mean Survival Time	
1)	UNTREATED	9/9	10.0 ± 0	
	**R_x LYT 1.2	4/8	19.6 ± 8.9 (xxx, xxxx)	S
2)	UNTREATED	10/10	11.3 ± 4.5	
	xxR_x LYT 2.2	4/4	9.5 ± 1.5	oNS
	**R_x THY 1.2	6/6	10.7 ± 3.5	NS
3)	UNTREATED	6/6	7.3 ± 2.1	
	**R_x THY 1.2	5/5	9.0 ± 1.6	NS

* $(C57XA)_{f1}$ Hybrid Mice + 1000 R ^{60}CO + 5 x 10^6 a bone marrow and spleen cells

** Treatment consisted of one injection of 0.1 ml of a 1:100 dilution of the monoclonal antibody under study

xxx Standard Deviation

xxxx Significant 0.05

o Not significant

TABLE X

THE EFFECT OF THREE IN VIVO TREATMENTS WITH MONOCLONAL ANTIBODY ON DAYS 1, 3, AND 7 POST-TRANSPLANT ON MORTALITY FROM ESTABLISHED ACUTE GVHD

Experiment	Group*	21 Day Mortality	Mean Survival Time
1)	UNTREATED	9/9	12.8 ± 4.5*** xxxx
	**R_x THY 1.2	9/9	18.4 ± 5.9 NS
2)	UNTREATED	15/15	11.1 ± 2.2
	R_x LYT 2.2	9/9	14.8 ± 4.0 NS
3)	UNTREATED	9/9	10.1 ± 2.1
	R_x LYT 1.2	9/9	10.8 ± 3.2 NS

* $(C57XA)_{f1}$ Hybrid Mice + 1000 R ^{60}CO + 5 x 10^6 a bone marrow and spleen cells

** Treatment consisted of three injections of 0.1 ml of a 1:100 dilution of the monoclonal antibody under study

xxx Standard Deviation

xxxx Not Significant

transplantation all died of acute GVHD with a mean survival time of 12.8 ± 4.5 days. The nine mice receving three 0.1 ml injections of monoclonal antibody directed against the THY 1.2 antigen all died of acute GVHD with a mean survival time of 18.4 ± 5.9 days. Although mean survival time was increased in the treated groups the increase was not significant and overall 21 day survival was not improved. In experiment 2 fifteen untreated mice all died of acute GVHD with a mean survival time of 11.2 ± 2.2 days. Nine mice treated with monoclonal antibody to the LYT 2.2 antigen all died with a mean survival time of 14.8 ± 4.0 days. Again mean survival time was increased in the treated group but not significantly and overall survival was unchanged. In the third experiment shown in Table X all untreated mice died of acute GVHD with a mean survival time of 10.1 ± 2.1 days. Mice treated 3 times post-transplantation with monoclonal antibody directed against the LYT 1.2 antigen showed no increased survival with all mice dying with a mean survival time of 10.8 ± 3.2 days.

Our studies involving the treatment of already established acute GVHD have to this point in time been discouraging. Only one approach seemed to have a positive effect. A single treatment 4 days post-transplantation with monoclonal anti-LYT 1.2 reduced the 21 day acute GVHD mortality from control levels of 100% to 50% and increased the mean survival time from 10 to 20 days. However, single treatments with other monoclonal antibodies were unsuccessful. In addition multiple treatments with monoclonal anti LYT 1.2 were ineffective. The fact that a single dose of monoclonal anti LYT 1.2 on day 4 is somewhat effective while multiple doses on day 1, 3 and 7 are ineffective implies that time of administration of the monoclonal antibody therapy may be of utmost importance. This approach is currently under investigation in our laboratory.

We have also investigated the effects of monoclonal antibodies directed against T-cell antigens on the incidence of chronic GVHD mortality. Table XI shows the combined results of several experiments evaluating the effect of a single *in vitro* pretreatment with monoclonal antibodies on the chronic GVHD inducing potential of A strain bone marrow cells. The *in vitro* pretreatment was with a final dilution of 1:300 plus complement. Thirty-two lethally irradiated $(C57XA)F_1$ mice given untreated A strain bone marrow cells showed 63% chronic GVHD mortality by 60 days post-transplant and 78% chronic GVHD mortality at 90 days post-transplant. Mice receiving bone marrow cells pretreated *in vitro* with THY 1.2 plus complement showed 41% and 50% chronic GVHD mortality at days 60 and 90, respectively. All 9 lethally irradiated $(C57XA)F_1$ mice receiving A strain bone marrow cells pretreated with LYT 1.2 monoclonal antibody plus complement died of chronic GVHD by day 60. However, only 1 of 18 $(C57XA)F_1$ mice receiving A strain bone marrow cells pretreated with LYT 2.2 antibody died of chronic GVHD by day 90. Thus, it appears from our results that pretreatment of A strain bone marrow cells with monoclonal antibody directed against the THY 1.2 antigen can reduce the incidence of chronic GVHD mortality. Moreover, pretreatment with monoclonal antibody to the LYT 2.2 antigen almost eliminated chronic GVHD mortality in 18 recipient mice.

Table XII shows the combined results of several experiments studying the effectiveness of monoclonal antibodies to T-cell antigen as therapeutic agents for established chronic GVHD. In this series of experiments the percentage chronic GVHD mortality in 98 control $(C57XA)F_1$ mice receiving untreated A bone marrow cells was 83%, 87% and 93% at 60 days, 90 days and

TABLE XI

THE EFFECT OF A SINGLE IN VIVO PRETREATMENT WITH MONOCLONAL ANTIBODIES DIRECTED AGAINST T-CELL ANTIGENS ON THE CHRONIC GVHD POTENTIAL OF A STRAIN BONE MARROW CELLS

Lethally Irradiated Recipient Strain	Number	Treatment*	% Chronic GVHD Mortality 60 Day	90 Day
$(C57XA)_{F1}$	32	A BM Cells	20/32 (63%)	25/32 (78%)
$(C57XA)_{F1}$	24	A BM Cells R_X THY 1.2 + C**	10/24 (41%)	12/24 (50%)
$(C57XA)_{F1}$	9	A BM Cells R_X LY 1.2 + C	9/9 (100%)	--
$(C57XA)_{F1}$	18	A BM Cells R_X LY 2.2 + C	1/18	1/18

* Treatment with a final dilution of 1:300

** Complement

TABLE XII

THE EFFECT OF A SINGLE IN VIVO PRETREATMENT WITH MONOCLONAL ANTIBODY DIRECTED TO T-CELL ANTIGENS ON MORTALITY FROM ESTABLISHED CHRONIC GVHD

Lethally Irradiated Recipient Strain	Number	Treatment	% Mortality 60 Day	% Mortality 90 Day	% Mortality Year
$(C57XA)_{F1}$	98	A BM Cells	81/98 (83%)	86/98 (88%)	91/98 (93%)
$(C57XA)_{F1}$	67	A BM Cells + R_x THY 1.2 day 18	48/67 (71%)	48/67 (71%)	51/67 (76%)
$(C57XA)_{F1}$	11	A BM Cells + R_x THY 1.1 day 18	9/11 (82%)	9/11 (82%)	11/11 (100%)
$(C57XA)_{F1}$	14	A BM Cells + R_x LYT 2.2 day 18	12/14 (86%)	12/14 (86%)	12/14 (86%)
$(C57XA)_{F1}$	6	A BM Cells + R_x LYT 1.2 day 18	6/6 (100%)	--	--

1 year, respectively. Sixty-seven mice receiving untreated A bone marrow cells plus an injection of 100 µl of a 1:100 dilution of monoclonal antibody 18 days post-transplant showed 70%, 71%, and 76% mortality at the 3 time points. The slight reduction in chronic GVHD mortality seen in these mice was not observed in 11 $(C57XA)_{F1}$ mice post-treated with monoclonal antibody directed to the THY 1.1 antigen (an antigen not present on the donor cells). $(C57XA)_{F1}$ mice treated 18 days post-transplant with monoclonal antibody to the LYT 1.2 or LYT 2.2 showed control levels of chronic GVHD mortality. Thus, it appears from our results that a slight therapeutic effect can be seen when monoclonal anti-THY 1.2 is used. However, the effect is minimal. The other antisera employed were unsuccessful in our model system.

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